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Induction of antioxidant enzymes in murine podocytes precedes injury by puromycin aminonucleoside

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Induction of antioxidant enzymes in murine podocytes precedes injury by puromycin aminonucleoside.

Background. An imbalance between the generation of reactive oxygen species (ROS) and antioxidant defense mechanisms has been suggested to play an important role in podocyte injury in nephrotic syndrome. Experimental nephrotic syndrome induced by injection of puromycin aminonucleoside (PAN) into rats is a well-established model of nephrotic syndrome, and can be largely prevented by pretreatment with antioxidant enzymes (AOE), suggesting that podocyte injury may be mediated by ROS.

Methods. To test the hypothesis that PAN-induced podocyte injury is modulated in part by podocyte antioxidant defenses, we analyzed AOE activities, lipid peroxidation products, and relative ROS levels in podocytes using our recently reported in vitro model of PAN-induced podocyte injury.

Results. PAN treatment induced early increases in both podocyte hydrogen peroxide and superoxide and later increases in lipid peroxidation products. Compared to baseline activities, PAN also induced significant changes in the major cellular AOE activities (maximum increases of 151% for catalase, 134% for superoxide dismutase, and 220% for glutathione peroxidase vs. time-matched controls). These changes largely preceded the development of extensive podocyte process retraction and actin filament disruption, which was maximal at 7 days.

Conclusion. These results demonstrate that (*1*) PAN treatment induces significant early changes in podocyte ROS, (*2*) podocytes can mount an antioxidant defense against oxidant stress, and (*3*) this protective response is initiated prior to the development of extensive oxidant-induced podocyte structural injury. These findings suggest that enhancement of podocyte AOE activities represent a potential therapeutic target to protect from or ameliorate podocyte injury during nephrotic syndrome.

Key words: nephrotic syndrome, reactive oxygen species, catalase, superoxide dismutase, glutathione peroxidase.

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Nephrotic syndrome is a common kidney disease in adults, and among the most common kidney diseases seen in children, with an estimated incidence of two to seven new cases annually per 100,000 children [1]. Nephrotic syndrome is characterized by morphologic changes in the visceral epithelial cells (podocytes) of the kidney glomerulus. The podocyte cell body and primary cell processes are suspended in the urinary space of the glomerulus, and finer secondary and tertiary processes attach the cell to the underlying glomerular basement membrane and interdigitate with processes from adjacent podocytes. The gaps between these interdigitating processes are spanned by a specialized cell-cell junction, the slit diaphragm. During development of nephrotic syndrome, this elaborate morphology of the podocyte is dramatically altered, resulting in changes that include cell swelling, retraction, and effacement (spreading) of foot processes, vacuole formation, formation of occluding junctions with displacement or disruption of the slit diaphragms, and foot process detachment from the glomerular basement membrane [2–6]. The effacement of foot processes correlates closely with the development of leakage of protein across the glomerular filtration barrier resulting in proteinuria [3, 5, 7], and is the characteristic ultrastructural finding for nephrotic syndrome.

Recently, positional cloning studies have identified several gene mutations responsible for certain rare forms of nephrotic syndrome, and mouse gene knockout studies have identified a number of podocyte proteins whose functional absence leads to the development of nephrotic syndrome. These studies have clearly established the importance of specific podocyte proteins, especially proteins shown to reside in the slit diaphragm, for glomerular function [reviewed in 8, 9]. However, far fewer data are available regarding the molecular mechanisms that are responsible for the changes in podocyte structure and function in the majority of patients with idiopathic and acquired forms of nephrotic syndrome.

One potential mechanism for these changes has been suggested by numerous studies in animal models of

nephrotic syndrome that have indirectly linked oxidant injury of podocytes with the subsequent development of foot process effacement and nephrotic syndrome. The most commonly used experimental model of nephrotic syndrome is the injection of puromycin aminonucleoside (PAN) into rats, a treatment that leads to development of foot process effacement and proteinuria within 7 to 10 days [6, 10]. The development of nephrotic syndrome has been associated with an early and transient induction of glomerular reactive oxygen species (ROS) [11], a decrease in glomerular antioxidant enzyme (AOE) activity [12], and accumulation products of oxidative damage to membranes (lipid peroxides) in the renal cortex [13], glomeruli [14, 15], and urine [13–15]. Conversely, pretreatment of animals with ROS scavengers [16–18] or the iron chelator deferoxamine (which prevents iron-catalyzed production of ROS) [19] prior to induction of experimental nephrotic syndrome resulted in either marked attenuation or complete prevention of both clinical and histologic disease. One major weakness of all of these studies, however, is that there have never been any measurements of either ROS or AOE activities or protein levels directly within podocytes, the glomerular cell most critically involved in the pathogenesis of nephrotic syndrome.

Based on these findings, we hypothesized that oxidant-induced podocyte injury may be a critical final common pathway for the development of nephrotic syndrome, and more specifically that PAN-induced podocyte injury is mediated by ROS and modulated by podocyte antioxidant defenses. To test this hypothesis, we designed studies to specifically measure, in podocytes, the activities and protein expression of the major cellular AOE, catalase (CAT), superoxide dismutase (SOD), and glutathione peroxidase (GPx), as well as measuring the relative amounts of the major cellular ROS, hydrogen peroxide and superoxide, and a product of lipid peroxidation, malondialdehyde (MDA), produced within podocytes both before and following PAN-induced podocyte injury.

METHODS

Cell culture

A clonal cell line of conditionally-immortalized murine podocytes (MPC-5) isolated from the "Immortomouse" was the kind gift of Dr. Peter Mundel [20]. Podocyte cells were grown and induced to differentiate as previously described [21]. All cultured podocytes used in this study were allowed to differentiate without subcultivation for 8 to 10 days prior to treatment. Cells were seeded in 75 cm^2 flasks (1×10^6 cells/flask), 6-well culture dishes (3×10^5 cells/well), or 96-well culture dishes (1×10^3 cells/well) for differentiation and treatment. Treatment consisted of the addition of PAN (5 μg/mL from 1000× aqueous stock solution) or vehicle (sterile water) in fresh medium, and culture for various time periods in the presence of PAN.

Cell viability assay

Podocytes differentiated in 96-well culture dishes were sham- or PAN-treated for specific times, and cell viability was assayed by 3-[4,5] dimethylthiazol-2,5-diphenyltetrazolium bromide (MTT) assay as previously described [22]. Briefly, 50 μL of 50 mg/mL MTT was added to wells containing treated cells under 200 μL of medium, and cells were incubated 4 hours under culture conditions. Medium containing MTT was removed, and 150 μL of dimethyl sulfoxide (DMSO) was added to all wells, and plates were read immediately at 570 nm. Wells containing no cells (but containing medium and MTT over the incubation period) were used to blank the plate reader.

Preparation of podocyte extracts

After treatments, medium was removed and cells were washed with warm phosphate-buffered saline (PBS). Cells were then collected by addition of trypsin solution (Gibco-BRL, Rockville, MD, USA), brief incubation, and centrifugation at 2000*g* for 5 minutes. The number of cells in suspensions were determined by counting cells in an aliquot in a hemocytometer prior to centrifugation. Cells were washed with PBS, centrifuged again, and lysed on ice with agitation for 15 minutes in cold hypo-osmotic buffer [50 mmol/L potassium phosphate, 0.1 mmol/L ethylenediaminetetraacetic acid (EDTA), and 0.1% Triton X-100, pH 7.8]. The amount of lysis buffer used was adjusted to a ratio of 5×10^6 cells/mL buffer. Extracts were then centrifuged at 12,000*g* for 15 minutes at 4°C. Supernatants were stored at −70°C.

ROS assays

Podocytes differentiated in 6-well dishes were treated with vehicle alone or with 5 μg/mL of PAN for 1, 3, 5, 7, or 12 hours, or for 1, 3, 5, or 7 days. Cells were then loaded with either 2 μmol/L 5-(and 6-)chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) or 3 μmol/L dihydroethidium (DHE) dissolved in medium without serum for 30 minutes. These compounds are relatively specific for determination of hydrogen peroxide [oxidation of CM-H_2DCFDA to carboxydichlorofluorescein (CDCF)] or superoxide (oxidation of DHE to ethidium), respectively [23, 24]. Cells were subsequently washed with Hank's balanced salt solution (HBSS) buffer (10 mmol/L HEPES, 150 mmol/L NaCl, 5 mm KCl, 1 mmol/L $MgCl_2$, and 1.8 mmol/L $CaCl_2$) and fluorescence was measured at 520 nm after excitation at 485 nm (CDCF), at 612 nm after excitation at 518 nm (ethidium bound to DNA), or at 430 nm after excitation at 410 nm

(DHE) with an Ascent Fluoroskan plate reader (MTX Lab, Inc., Vienna, VA, USA).

Thiobarbituric acid assay of podocyte MDA

Podocytes differentiated in 6-well dishes were treated with vehicle alone or with 5 μg/mL of PAN for 0, 1, 3, 5, or 7 days. Cells were washed with ice-cold PBS and then scraped into 500 μL per well of ice-cold RIPA buffer [50 mmol/L Tris-Cl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 5 μg/mL each aprotinin and leupeptin, 0.1% Triton X-100, 1% sodium deoxycholate, and 0.1% sodium dodecyl sulfate (SDS)] and homogenized by sonication four times for 5 seconds each on ice. Homogenates were centrifuged at 14,000*g* for 20 minutes, an aliquot of the supernatant was removed for protein assay (see below), and trichloroacetic acid was added to the remaining supernatant to a final concentration of 6% (wt/vol). After a 15-minute incubation on ice, protein precipitates were pelleted by centrifugation at 3000*g* for 15 minutes at 4°C. To determine the concentration of MDA in supernatants, an equal volume of 0.67% (wt/vol) thiobarbituric acid was added to an aliquot of each supernatant and samples were held in a boiling water bath for 10 minutes, cooled, and absorbance read at 530 nm. MDA concentrations were calculated from a standard curve of various concentrations of 1,1,3,3-tetramethoxypropane subjected to the same assay.

AOE activity assays

Cu-Zn- and Mn-SOD activities were measured by oxidation of 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo[c]fluorene in aqueous alkaline solution to yield a chromophore with maximum absorbance at 525 nm (Bioxytech® SOD-525™) (Oxis Research, Portland OR, USA). The SOD activity was expressed in units (U) defined as the activity that doubled the auto-oxidation rate of a control blank containing 5,6,6a,11b-tetrahydro-3,9,10-trihydroxybenzo[c]fluorene.

CAT activity was measured by consumption of hydrogen peroxide, as determined by absorbance at 240 nm. CAT activity was expressed in units (U) defined as the k value of the enzyme reaction at 25°C where:

$$k = (1/(t_2 - t_1))(\ln(OD_1/OD_2))$$

where OD = absorbance of H_2O_2 and t = time in seconds.

GPx activity was measured by the conversion of the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) to $NADP^+$ by glutathione reductase, the enzyme that reduces the glutathione oxidized by GPx, as determined by the decrease in absorbance of the substrate, NADPH, at 340 nm (Glutathione Peroxidase Assay Kit) (Cayman Chemical, Ann Arbor, MI, USA). GPx activity was expressed in units (U) defined as the amount of GPx required to oxidize 1.0 nmol of NADPH to $NADP^+$ at 25°C/min/mL.

Phase-contrast and phalloidin-labeled fluorescence microscopy

Cells grown on coverslips and treated were washed briefly in warm PBS and fixed in 3.7% paraformaldehyde in PBS, pH 7.4, for 30 minutes. Cells were lysed by addition of an equal volume of 0.5% Triton X-100 in PBS to the fixation solution for 5 minutes. After removal of the fixative, cells were washed with 0.05% Tween-20 in PBS (T-PBS) and incubated with Texas Red-conjugated phalloidin (Molecular Probes, Eugene, OR, USA) diluted 1:250 in T-PBS for 30 minutes. Coverslips were washed and mounted in ProLong Antifade (Molecular Probes) according to the manufacturer's instructions.

Phase-contrast and fluorescence microscopy was performed using an inverted fluorescence microscope (DMIRE-2) (Leica Microsystems, Inc., Bannockburn, IL, USA) and images were collected with a monochrome cooled charged coupling device (CCD) camera (Retiga 1300C; Retiga, Millersville, MD, USA) using OpenLab 3.1.4 software (Improvision, Inc., Lexington, MA, USA). Images were adjusted for contrast and gain in Adobe Photoshop version 7.0.1.

Protein assay

The Bio-Rad DC Protein Assay (Bio-Rad, Hercules, CA, USA) microplate protocol was used to quantify total protein in samples. Lysis and SDS-polyacrylamide gel electrophoresis (PAGE) sample buffers were used to prepare albumin protein standards to normalize for the effect of buffer components on the assay results.

Statistics

Results were analyzed for statistical significance using Statview version 4.57 software (Abacus Concepts, Berkeley, CA, USA) by the unpaired, two-tailed *t* test. Comparisons with P values < 0.05 were considered significant, and P values < 0.01 were separately noted.

RESULTS

Podocyte viability decreases after PAN

The viability of cultured, differentiated, murine podocytes treated with 5 μg/mL PAN for various times or vehicle for various times was measured by MTT assay. Podocyte viability after PAN treatment was not significantly different from vehicle-treated, time-matched controls except after 7 days of treatment, when absorbance in PAN-treated samples was 70 ± 8% of control values (Fig. 1).

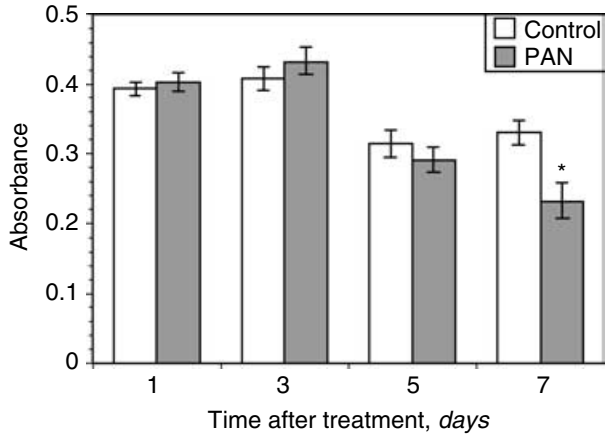

Fig. 1. Podocyte viability decreases after puromycin aminonucleoside (PAN) treatment. Results of absorbance measurements at 570 nm, normalized for background, after 3-[4, 5] dimethylthiazol-2,5-diphenyl-tetrazolium bromide (MTT) assay of cultured podocytes treated with 5.0 μg/mL PAN for 1, 3, 5, or 7 days. Podocyte viability at 7 days after treatment was significantly decreased by PAN treatment compared to time-matched controls ($N = 8$; $\chi \pm$ SEM). $^*P < 0.05$ vs. time-matched controls.

PAN induces podocyte process retraction and actin filament disruption

Cultured podocytes prior to treatment displayed a characteristic flattened phenotype with numerous cell processes (Fig. 2A), and prominent central actin stress fibers (Fig. 2B). Treatment of podocytes with 5.0 μg/mL PAN resulted in a progressive retraction of processes by 5 days after treatment (Fig. 2G) and subsequent detachment of some podocytes from the underlying substrate. Initial disruption of the central actin stress fibers was first detectable after 1 day of PAN treatment (Fig. 2D), and by 7 days after treatment actin stress fibers were almost entirely absent (Fig. 2J).

PAN treatment induces increases in both podocyte hydrogen peroxide and superoxide

Fluorescence was measured in podocytes treated with PAN for various periods and then loaded with the cell-permeant fluorescent dyes DHE and CM-H_2DCFDA. When DHE is oxidized to ethidium in the cytoplasm it can enter the nucleus and intercalate with cellular DNA, resulting in a shift from blue fluorescence (DHE) to red (ethidium). The acetoxymethyl ester groups of CM-H_2DCFDA are removed by intracellular esterases, and intracellular ROS oxidize this compound to CDCF, which is more fluorescent (oxidation) and less able to cross the cell plasma membrane (de-esterification). Increases in the ratio of ethidium to DHE fluorescence and in fluorescein fluorescence derived from CM-H_2DCFDA are thus measures of increases in ROS, more specifically of increases in cellular hydrogen peroxide (CDCF) and superoxide (ethidium) [24].

A significant increase in the mean fluorescein fluorescence in cells treated with PAN and subsequently loaded with CM-H_2DCFDA was only observed at 3 hours after PAN treatment, and the magnitude of this change was relatively small (127% of $t = 0$ value) (Fig. 3). This result indicates that hydrogen peroxide generation was only transiently and modestly increased in podocytes in response to PAN treatment. No significant changes in hydrogen peroxide in PAN-treated podocytes were detected (compared to time-matched controls) at later time points (1, 3, 5, or 7 days after PAN treatment) (data not shown).

In contrast, an increase in the ratio of mean ethidium to DHE fluorescence was observed at all time points tested, although this change was only statistically significant at 7 and 12 hours after PAN treatment (546% and 551% of $t = 0$ value, respectively) (Fig. 4). The fluorescence observed in cells treated with PAN remained elevated for at least 12 hours, indicating that the increase in the rate of superoxide generation in podocytes, unlike the modest and transient increase in hydrogen peroxide, was more persistent. However, no significant changes in superoxide in PAN-treated podocytes were detected (compared to time-matched controls) at later time points (1, 3, 5, or 7 days after PAN treatment) (data not shown).

PAN treatment induces increases in podocyte MDA

The concentration of MDA was measured by thiobarbituric acid assay in deproteinated extracts of vehicle alone and PAN-treated podocytes at 1, 3, 5, and 7 days after treatment. By 5 days after PAN treatment, the amount of MDA normalized to the quantity of total cellular protein had increased significantly to 2.42 ± 0.26 nmol/μg protein, and increased further by 7 d after treatment to 5.71 ± 1.14 nmol/μg protein (509% and 400% of time-matched, vehicle-treated control values, respectively, see Figure 5). These results demonstrate that PAN treatment significantly increased the amount of a product of lipid peroxidation (MDA) in cultured podocytes.

PAN treatment induces podocyte AOE activities

The baseline activities of the three major cellular AOE (SOD, CAT, and GPx) were measured in cultured, differentiated, murine podocytes (Table 1). Measurable amounts of all three enzymes were detected.

The amount of CAT activity in sham-treated control cells did not change significantly from its mean baseline value at any time point tested, although there was a trend toward decreased activity with increasing time after sham treatment (Fig. 6). In contrast, by 3 days after treatment with 5 μg/mL PAN, CAT activity increased significantly to 35.2 ± 2.4 ($\times \pm$ SEM) mU/500 k podocytes (151% of time-matched controls) ($P < 0.01$), and remained significantly increased over the remaining 4-day treatment period

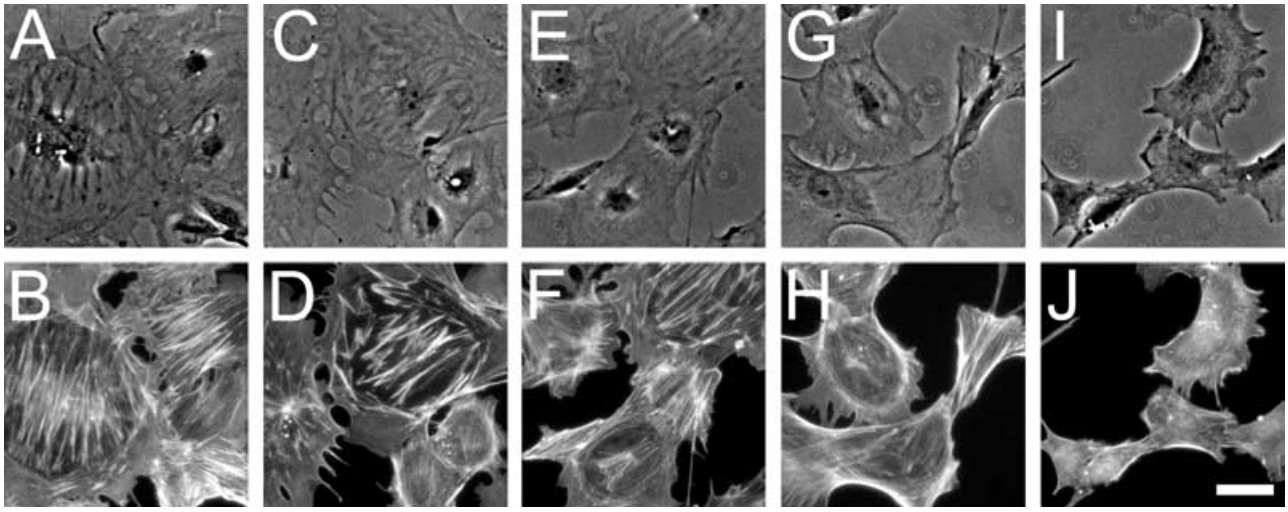

Fig. 2. Puromycin aminonucleoside (PAN) induces podocyte process retraction and actin filament disruption. Phase-contrast (*A*, *C*, *E*, *G*, and *I*) and fluorescence (*B*, *D*, *F*, *H*, and *J*) micrographs of cultured podocytes are shown following treatment for 0 (A and B), 1 (C and D), 3 (E and F), 5 (G and H), or 7 days (I and J) with 5 μg/mL PAN. Podocytes retracted and lost processes by 5 d of treatment, while actin filament disruption could be detected as early as 1 day after addition of PAN.

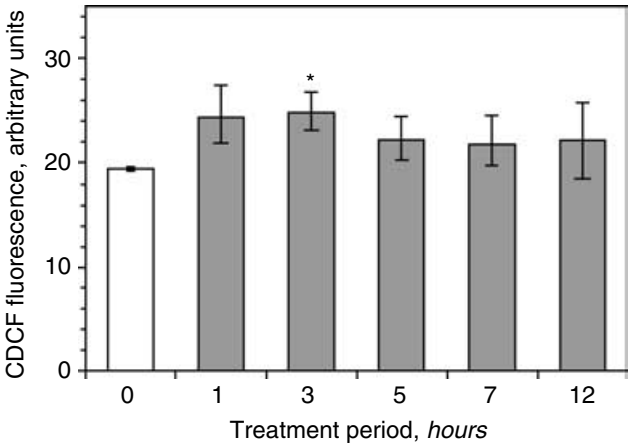

Fig. 3. Puromycin aminonucleoside (PAN) treatment induces an increase in podocyte hydrogen peroxide. The results of fluorescence measurements of carboxydichlorofluorescein (CDCF) at 520 nm after excitation at 485 nm of cultured podocytes treated with 5.0 μg/mL PAN for various times and a t = 0 control and then loaded with 5-(and 6-)chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate (CM-H_2DCFDA) for 30 minutes. PAN treatment (■) for 3 hours induced a modest and transient increase in fluorescence compared to cells that did not receive PAN treatment (□) ($N = 3$; $\chi \pm$ SEM). $^*P < 0.05$ vs. t = 0.

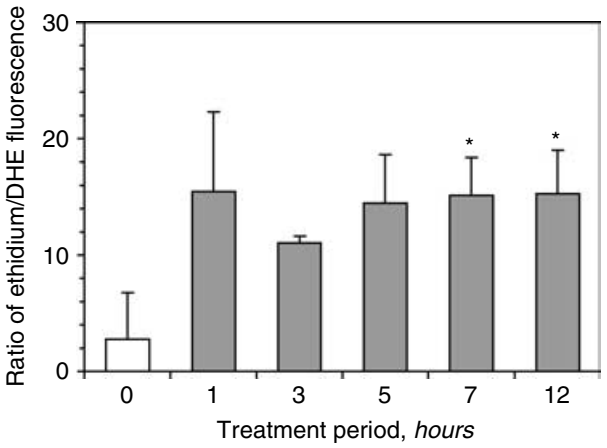

Fig. 4. Puromycin aminonucleoside (PAN) treatment induces an increase in podocyte superoxide. The ratio of fluorescence measurements at 612 nm after excitation at 518 nm (ethidium) and at 430 nm after excitation at 410 nm [dihydroethidium (DHE)] of cultured podocytes treated with 5.0 μg/mL PAN for various times and a t = 0 control and then loaded with DHE for 30 minutes. PAN treatment (■) for 7 and 12 hours induced a significant increase in the ratio of ethidium to DHE fluorescence compared to cells that did not receive PAN treatment (□) ($N = 3$; $\chi \pm$ SEM). $^*P < 0.05$ vs. t = 0.

(Fig. 6). The greatest increase in CAT activity (197% of time-matched controls) ($P < 0.01$) was observed at 7 days after PAN treatment.

The amount of SOD activity in sham-treated control cells increased significantly (approximately 140% of t = 0 controls) ($P < 0.05$) to a mean value of approximately 50 U/500 k podocytes at 5 and 7 days after sham treatment (Fig. 7). Compared to these values, treatment with PAN induced a significant increase in podocyte SOD activity to 66.7 ± 5.9 (SE) U/500 k podocytes (134% of time-matched controls ($P < 0.05$) only at 7 days after treatment, and this increase was also the largest detected (Fig. 7).

The amount of GPx activity in sham-treated control cells increased significantly (188% of time-matched controls) ($P < 0.05$) at 1 day from its mean baseline value to 48.0 ± 2.4 U/500 k podocytes, but was not different from the day 0 value at any other time point tested (Fig. 8). In response to PAN treatment, GPx activity increased significantly (220% of time-matched controls) ($P < 0.01$) at day 3 of treatment to 62.7 ± 4.1 U/500 k

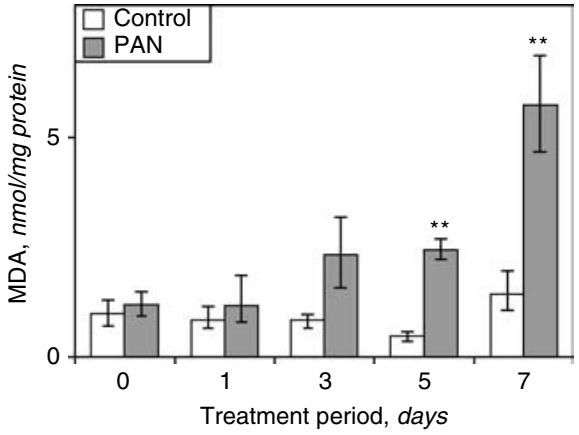

Fig. 5. Puromycin aminonucleoside (PAN) treatment induces an increase in podocyte malondialdehyde (MDA). The amounts of MDA normalized to total protein of cultured podocytes treated with vehicle alone or 5.0 μg/mL PAN for various times. Treatment with PAN for both 5 and 7 days induced a significant increase in podocyte MDA compared to cells treated with vehicle alone ($N = 5$; $\chi \pm$ SEM). $^{**}P < 0.01$ vs. time-matched controls.

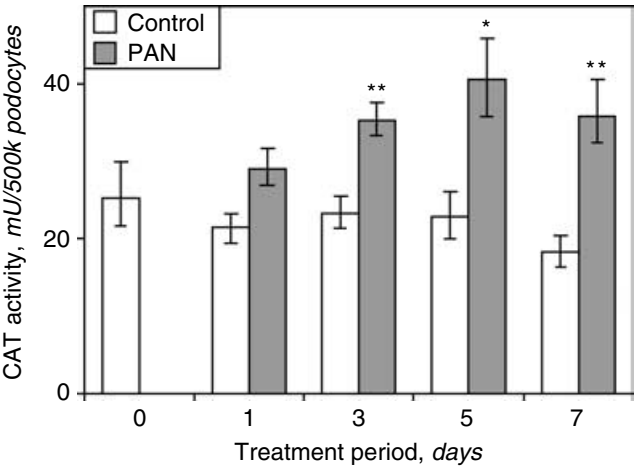

Fig. 6. Puromycin aminonucleoside (PAN) treatment induces podocyte catalase (CAT) activity. The results of CAT enzyme activity assays in cultured podocytes treated with vehicle alone or with 5.0 μg/mL PAN for various times and a t = 0 control. PAN treatment induced a significant increase in CAT activity compared to time-matched sham-treated controls at 3, 5, and 7 days after PAN treatment. Enzyme activity is expressed as k value ($N = 7$; $\chi \pm$ SEM). $^{*}P < 0.05$; $^{**}P < 0.01$ vs. time-matched control.

Table 1. Baseline antioxidant enzyme activities of differentiated podocytes

Enzyme	Activity/mg protein[a]	Activity/500 k podocytes[b]
SOD	47.3 ± 6.2 U	35.7 ± 3.8 U
CAT	35.6 ± 3.7 mU	25.2 ± 4.7 mU
GPx	34.2 ± 6.7 U	25.5 ± 5.3 U

Abbreviations are: SOD, Mn- and Cu-Zn-superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase.

[a]Activity normalized to total protein content ($N = 8$, $\chi \pm$ SD); [b]Activity normalized to podocyte cell number ($N = 8$, $\chi \pm$ SD).

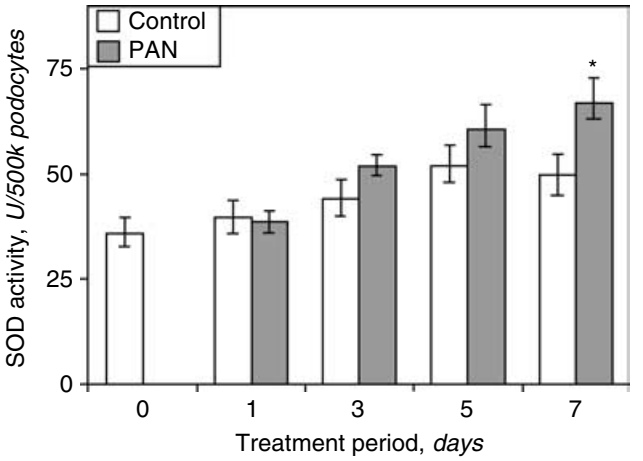

Fig. 7. Puromycin aminonucleoside (PAN) treatment induces podocyte superoxide dismutase (SOD) activity. The results of SOD enzyme activity assays in cultured podocytes treated with vehicle alone or with 5.0 μg/mL PAN for 1, 3, 5, or 7 days and a t = 0 control. PAN treatment induced a significant increase in SOD activity only after 7 days of treatment compared to time-matched controls ($N = 8$; $\chi \pm$ SEM). $^{*}P < 0.05$ vs. time-matched control.

podocytes, but was not different from time-matched controls at any other time points tested.

Together these results reveal that all three major cellular AOE have detectable activities in untreated cultured podocytes. In addition, the activity of each of these AOE increased following PAN-induced ROS generation in these cells, although the amount of increase in AOE activity and the time course of induction varied between different enzymes after PAN exposure.

DISCUSSION

There is abundant indirect evidence suggesting that oxidant injury to podocytes may have an important role in the development of the structural and functional alterations that occur in podocytes during nephrotic syndrome. Most of the data on AOE activities, however, have been derived from measurements of glomerular and renal cortical samples [11, 25], permitting only indirect measurements of the AOE capacity of podocytes to protect themselves from oxidant injury. In the current study we report for the first time the direct measurement of the activities of the three major cellular AOE in cultured murine podocytes, and that podocyte injury by PAN results in induction of podocyte ROS as well as induction of podocyte AOE activities. While the timing and extent of AOE induction was variable, induction of a protective antioxidant response was initiated prior to the development of extensive podocyte structural and actin cytoskeletal injury. These findings suggest that enhancement of podocyte AOE activities represent a potential therapeutic target to protect from or ameliorate podocyte injury during nephrotic syndrome.

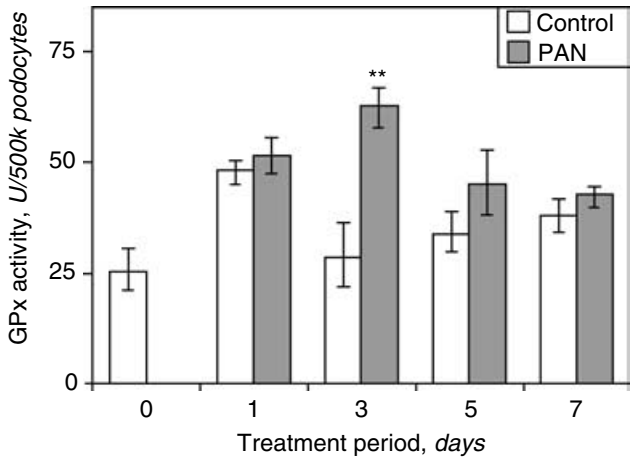

Fig. 8. Puromycin aminonucleoside (PAN) treatment induces podocyte glutathione peroxidase (GPx) activity. The results of GPx enzyme activity assays in cultured podocytes treated with vehicle alone or with 5.0 μg/mL PAN for 1, 3, 5, and 7 days and a t = 0 control. PAN treatment induced a significant increase in GPx activity compared to time-matched controls only at 3 days after PAN treatment ($N = 8$; $\chi \pm$ SEM). $^{**}P < 0.01$ vs. time-matched control.

Injection of PAN into rats is a well-described model system for the induction of experimental nephrotic syndrome [5, 26]. A number of reports have indirectly linked PAN-induced nephrosis with oxidant injury of podocytes, including detection of increased amounts of hydroperoxides in rat glomeruli after PAN injection [27–29], hydrogen peroxide generation in rat glomeruli in response to PAN that could be blocked by inhibitors of cytochrome P450 enzymes [30], and increases in glomerular lipid peroxidation products after PAN treatment [14, 31]. Increased lipid peroxidation has also been measured in glomeruli from humans with congenital nephrotic syndrome of the Finnish type [32]. Further evidence in support of this potential mechanism includes studies demonstrating that treatments commonly used to treat nephrotic syndrome in humans, such as glucocorticoids and other immunosuppressants, can increase glomerular AOE and protect against PAN-induced injury [12, 14], studies showing that AOE or antioxidants can ameliorate the effects of PAN [28, 29, 33, 34] and that inhibition of AOE exacerbates PAN nephrosis [35]. A metabolite of PAN has also previously been shown to be converted by glomeruli to hypoxanthine, a component of the xanthine/hypoxanthine pathway for the production of superoxide radicals [36]. However, this report also demonstrated that PAN itself, as well as other metabolites of PAN that were not converted to hypoxanthine, were equally toxic to cultured podocytes. These reports together provide strong indirect evidence that the PAN-induced injury to podocytes resulting in experimental nephrotic syndrome is mediated by ROS, but they do not clearly define either the mechanism or cell of origin of ROS generation in PAN nephrosis.

In contrast to these prior studies, in the present studies we observed, specifically in podocytes, significant increases in fluorescence from oxidized metabolites of both CM-H_2DCFDA (a measure of cellular hydrogen peroxide) and DHE (a measure of cellular superoxide), as well as significant increases in the amount of MDA (a measure of lipid peroxidation) and the activities of the three major cellular AOE in response to PAN-induced injury. The increases in fluorescence from the conversion of DHE to ethidium, primarily due to superoxide, were greater in magnitude and more persistent than the modest and transient induction of fluorescence derived from the oxidation of CM-H_2DCFDA (see Figs. 3 and 4), which is consistent with the previous results suggesting that PAN is converted to hypoxanthine, resulting in generation of superoxide. Although significant changes in either DHE and CM-H_2DCFDA in PAN-treated podocytes were only detected at times less than 24 hours after PAN treatment, the significant increases in the amount of MDA (a product of lipid peroxidation) measured 5 and 7 days after PAN treatment (see Fig. 5) demonstrated that PAN-induced generation of ROS continued throughout the 7-day treatment period. Comparably elevated MDA levels (2.93 nmol MDA/mg protein, 230% to 330% of controls) have previously been detected in glomeruli of PAN-treated rats [37], and increases in MDA have also been demonstrated in glomeruli of patients with congenital nephrotic syndrome of the Finnish type [32] and in red blood cells from patients with a variety of glomerular diseases. Our inability to detect the generation of ROS during a 30-minute incubation with ROS indicator fluorophores in the period from 1 to 7 days after PAN treatment suggests that the rate of ROS generation in podocytes exposed to PAN decreases after 12 hours but apparently remains high enough to lead to cumulative ROS-induced injury (increased MDA after 5 and 7 days of PAN treatment). Since the increases in CAT and GPx activities were most evident at 3 to 5 days after exposure to PAN, while the increase in SOD activity was only significant at 7 days after PAN treatment, the generation of hydrogen peroxide, possibly from superoxide, is likely to be the molecule that induces the most dramatic cellular defense response. It is notable that while the maximal induction of CAT and SOD activities occurred at a time that cultured podocytes had already developed clearly altered morphology (Fig. 2G and I) and disruption of actin filaments (Fig. 2H and J), the initial increases in podocyte CAT and GPx activities were observed prior to extensive changes in the morphology of the majority of cultured podocytes, and when only modest actin filament disruption was observed (Fig. 2E and F). These results are in contrast to a previously observed decrease in GPx and CAT activities in isolated rat glomeruli at 9 days after induction of PAN nephrosis, where no detectable increases in AOE were seen despite both an early (15 minutes) and

late (9 days) induction of ROS by PAN in these glomeruli [38]. Similarly, glomerular GPx levels have been reported to be decreased in both patients with focal segmental glomerulosclerosis and in rats with PAN-induced sclerosis [39]. This discrepancy between our results and earlier studies may indicate that isolated podocytes are capable of responding to oxidant stress by induction of AOE activities, but that the prior measurements of total glomerular AOE activities, in which other glomerular cells may have had decreased AOE activities, masked the AOE induction specifically within the podocytes. Alternatively, podocytes in vivo may be unable to effectively increase AOE activities in the functional milieu of the glomerulus where, in contrast to culture conditions, continuous blood perfusion and filtration are both occurring.

Our studies identified notably different podocyte AOE activities than have previously been estimated from analyses of intact glomeruli. For example, assuming there are approximately 300 podocytes per glomerulus [40], our measured mean CAT activity of 25 mU/500 k podocytes correlates to a derived value of 15 mU of podocyte CAT activity/1000 glomeruli, a value markedly higher than the previously reported 1 to 2 mU/1000 rat glomeruli [25]. Similarly, the SOD and GPx activities measured in cultured murine podocytes (21 U SOD and 15 U GPx per 300 k cultured podocytes) were considerably higher than the values described previously in rat glomeruli (3 U SOD and 4 U per 1000 rat glomeruli) [25]. There are several potential explanations for these discrepancies, including loss of glomerular AOE activities during the prolonged (>1 hour) procedures required for glomerular isolation compared to the immediate isolation of proteins from cultured cells, the presence of factors in glomeruli that may interfere with enzyme assays, blunting of podocyte AOE findings when analyzed as part of entire glomerular preparations, and species variation of AOE activities between mice and rats. Our use of an SOD enzyme assay kit using a new substrate also makes comparison of our findings with previous literature difficult, as the definition of enzyme activity units is based on relative differences between sample based oxidation and auto-oxidation of this substrate rather than reduction in cytochrome C absorbance in a xanthine/xanthine oxidase coupled system as previously reported [25]. Finally, it has also been reported that the CAT activity of primary cultures of mesangial cells increases after prolonged periods in culture [25], suggesting that the increased AOE activities found in conditionally immortalized murine podocytes may be a result of culture conditions.

Together our findings support the hypothesis that PAN acts via ROS to induce experimental nephrotic syndrome, and that podocytes express active AOE and can respond to oxidant injury by induction of CAT, SOD, and GPx activities. Furthermore, the induction of AOE activity prior to the development of extensive changes in podocyte morphology and actin cytoskeleton suggests that enhancement of podocyte AOE activities might represent a potential therapeutic target to protect from or ameliorate podocyte injury during nephrotic syndrome.

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